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# *In vitro* modeling of the blood-brain barrier: simplicity versus complexity

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**Abstract**

Proper understanding of blood-brain barrier (BBB) regulation is crucial to reduce/prevent its disruption during injury. Since high brain complexity makes interpretation of *in vivo* data challenging BBB studies are frequently performed using simplified *in vitro* models. Although such models represent an important and frequently employed alternative for investigation of BBB function and alterations, our ability to translate *in vitro* findings to *in vivo* situation remains sub-optimal. Consequently, despite the fact that our knowledge of the cellular and molecular mechanisms underlying BBB physiology and pathophysiology is constantly increasing, our ability to modulate barrier function remains virtually non-existent. Classical *in vitro* model systems have provided a wealth of knowledge until now, but it is now evident that newer *in vitro* models that are more representative of the *in vivo* situation are needed to further our understanding of barrier physiology.

This paper will provide an overview of the BBB cellular components and the most frequently used *in vitro* BBB model systems. I will discuss their advantages and disadvantages, as well as highlight recently developed models that more closely mimic the BBB *in vivo*.

**Introduction**

The blood-brain barrier (BBB) is a selective vascular barrier that maintains brain homeostasis. The BBB is dynamic and performs both passive and active features of the brain endothelium (1-3). It acts as a physical barrier due to presence of tight junction complexes between adjacent endothelial cells preventing the paracellular movement of molecular traffic across the BBB. By permitting or facilitating the entry of required nutrients while excluding or effluxing potentially harmful compounds via specific transport systems located on the endothelial luminal and abluminal membranes it also acts as a selective transport barrier. It is also a metabolic barrier due to the combination of intracellular and extracellular enzymes that inactivate many neuroactive and toxic compounds or metabolize peptides and ATP. All these tasks are critical since in their absence the homeostatic neuronal environment within the central nervous system would be significantly compromised leading to neuronal hyperactivation and malfunction (4, 5). It is therefore not surprising that alterations in BBB function have been associated with multiple pathologies and may represent a cause or consequence of disease progression with overall negative outcomes. On the other hand the ability of the BBB to efficiently exclude the entrance of foreign substances to the brain means that it also represents a formidable obstacle for drug entry and treatment of brain pathologies and other diseases (3, 4). Thus the conundrum for vascular biologists at present is how to maintain the barrier during injury but also facilitate selective opening to enable drug access.

*In vivo*, the BBB is a structure that is not easily accessible for study. The BBB exists as a complex microvascular network throughout the brain and therefore is difficult to isolate and target specific mechanisms. In addition, the complex cellular interactions within the brain parenchyma make it problematic to identify the specific contribution of the various cellular components and pathways to barrier function. Various animal injury models are used to study different diseases characterized by loss of BBB integrity but despite this fact, the induction and maintenance of the BBB is still poorly understood (3). Thus many researchers have developed *in vitro* model systems that represent highly

simplified models of the BBB to foster a more in depth understanding of the mechanisms and signaling pathways involved in barrier function. *In vitro* models are powerful cell-based assays and versatile experimental tools that can be used to study different features of the barrier in normal and pathological states. This review will compare various aspects of 2 and 3 dimensional models, their pros and cons and overall contribution to our knowledge of barrier induction and maintenance.

**Cellular components of the BBB**

The BBB is a sophisticated multicellular structure that is maintained by complex interactions between capillary endothelial cells, the basement lamina, pericytes and astrocytic endfeet processes (reviewed by (6)), which together with neurons and microglia form an organization referred to as the neurovascular unit (NVU).

The basement membrane is an essential part of the BBB that surrounds the capillaries thereby anchoring the cells in place and providing the link with the resident brain cells. Astrocytes, pericytes and endothelial cells themselves all contribute to the basement membrane that consists of structural proteins, collagens and specialised proteins (7). Disruption of the basement membrane can affect tight junction protein expression in the endothelium and has been associated with increased BBB permeability in various pathological conditions (8-10).

The endothelia of cerebral capillaries form the anatomic basis of the BBB in higher organisms (1, 11). Unlike the endothelium of other vascular beds, specialized cerebral microvessel endothelial cells have very low permeability due to the presence of highly organized junctional complexes called tight junctions (TJs). TJs ensure stringent regulation of CNS homeostasis by severe restriction of the paracellular diffusional pathway between the endothelial cells and substances and/or cells within the circulating blood (12). TJs are elaborate structures that span the apical region of the intercellular cleft of epithelial and endothelial barrier tissues functioning both as a "zipper" that effectively separates the apical and basolateral cell membranes enabling asymmetric distribution of membrane constituents, and a "fence" that limits paracellular permeability (12, 13). TJs respond quickly to intracellular signaling events and thus are highly dynamic structures that rapidly change expression, subcellular localization, and post-translational modifications, which in turn affect protein–protein interactions (13, 14). The effectiveness of endothelial TJs appears to be regulated via the intracellular scaffold proteins of the zonal occludens family that link the junctional molecules claudin and occludin to the cytoskeleton (7, 11, 15). Current evidence suggests that the claudins constitute the backbone of TJ strands and occludin plays a more permeability-regulating role by incorporating itself into the claudin-based strands (reviewed by (16)). The mechanism by which this occurs, and indeed the precise role(s) of occludin remain to be elucidated. An additional contribution of adherens junctions to stabilization of endothelial cell–cell interactions at the junctional zone has been recently reviewed (17, 18).

Notably, capillary endothelial cells do not intrinsically form a BBB but are induced by the CNS environment. Signalling among the BBB cells is thought to occur via soluble and solid phase factors and their cognate receptors. In this respect the participation of astrocytes has been most widely studied. Astrocytes are thought to induce the barrier phenotype of cerebrovascular endothelial cells during development through release of soluble factors such as vascular endothelial growth factor (VEGF),

TGF-β, IL-6, and nitric oxide (19-23). In general astrocytes are now accepted to play a decisive role not only in maintaining barrier properties of brain microcapillary endothelial cells (2, 24, 25), but also in direct control of cerebral blood flow (26, 27) and regulation of water homeostasis (28, 29).

In contrast, the contribution of pericytes to BBB phenotype has until recently remained more obscure (4). Pericytes were suggested to be involved in capillary contraction due to their expression of contractile proteins and intimate contact with endothelial cells (30), and in the retinal vasculature were shown to be important for retinal barrier function (31). Subsequent *in vitro* studies also suggested that pericytes enhance BBB function (32, 33). Very recently additional evidence was provided by some elegant *in vivo* studies. Platelet-derived growth factor (PDGF)-B, a high affinity ligand for the receptor tyrosine kinase PDGF-R expressed on peric and is critical for vascularization of the brain. Indeed disruption of the PDGF-B gene led to pericyte loss, endothelial hyperplasia, and prenatal lethality (34, 35). Using viable pericyte-deficient adult mouse models it has now been demonstrated that pericytes are necessary for the formation of the BBB as well as regulate functional aspects, including the formation of tight junctions and vesicle trafficking in CNS endothelial cells, astrocyte polarization and integration of cellular functions at the NVU (36, 37). Another group also showed that age-dependent vascular damage in pericyte-deficient mice precedes neuronal degenerative changes, learning and memory impairment, and the neuroinflammatory response (38). Thus, pericytes control key neurovascular functions that are necessary for both BBB maintenance and proper neuronal structure and function.

**Targeting the BBB to fight disease**

As stated above, cellular interactions and local release of factors, in addition to signals from circulating substances, have marked effects on TJ expression and barrier integrity. Thus it is not surprising that barrier disruption has been associated with a number of CNS pathologies including Parkinson's and Alzheimer's disease (5, 8), multiple sclerosis (39), hypoxia and ischemia (40), edema (41), tumorigenesis (42), epilepsy (43, 44) and glaucoma (45). The degree of dysfunction can range from mild and transient tight junction opening to chronic barrier breakdown. It is frequently unclear whether disease onset *per se* is triggered by barrier compromise, but its disturbance can contribute to, and exacerbate, the developing pathology (46).

The role of astrocytes and pericytes in BBB maintenance during brain injury or pathological progression has not been well addressed. Current research advocates that astrocytes support endothelial cell survival during hypoxia/ischemia and maintain barrier function (40, 47-49). Pericytes also seem to reduce paracellular flux across the barrier (32, 50). Reports of secretion of TGF-β (51, 52), erythropoietin (53), glial derived neurotrophic factor and neurotrophin (54) by astrocytes and/or pericytes during cerebral hypoxia/ischemia reveals the diversity of injury-regulated factors that might promote barrier maintenance and cell survival in endothelial cells during injury. However our own recent studies suggest that the ability of either cell type to maintain barrier function is profoundly and differentially modulated by severity and duration of the insult (50). For example during acute hypoxic insult astrocytes protected the endothelial barrier better than pericytes whereas during prolonged hypoxic insult pericytes were more protective than astrocytes, a discrepancy that may be attributable to

differences in modulation of specific signalling pathways and subsequent released factors such as VEGF (50). Undoubtedly direct but differential effects of injury processes on either astrocytes or pericytes must have significant consequences for BBB function and disease progression. Hence barrier maintenance is a complicated and highly regulated process involving multiple pathways. Until now the highly coordinated signalling mechanisms that exist between the specific cellular components of the BBB during injury remain obscure.

As early intervention offers the best possibility of reducing long-term disease progression and disability (3), the current challenge for the field is to develop better diagnostic methods to identify barrier disturbance and ways to therapeutically target the BBB to prevent barrier compromise. However it should not be forgotten that the opposite approach - selectively opening the tight junctions of the endothelium to facilitate drug delivery to the brain - is also an area of intense research. To achieve these aims we need to have a much better understanding of BBB function and regulation. Notably, since the contribution of individual cell types is crucial, identification and targeting of cell-specific mechanisms may gain better results than have been currently obtained using more global approaches. *In vitro* model systems have a significant role to play in this respect.

### *In vitro* model systems

*In vitro* modeling has greatly contributed to the BBB field. These simplified systems allow investigation and more accurate interpretation of experiments that are impossible, or at best highly complicated, to perform *in vivo*. Such models have increased our understanding of how influx and efflux of endogenous substances are controlled. Furthermore they have provided insight into the contribution of individual cell types to specific functions of barrier regulation, physiological as well as pathological processes. The fact that they are versatile, relatively easy to implement and inexpensive compared to *in vivo* models also makes them a frequently employed resource.

However *in vitro* model systems are unable to fully recreate all aspects of the *in vivo* situation and are limited predominantly by the type of brain endothelial cells used. Immortalized cell lines are more frequently utilized (due to the difficult isolation procedures of primary cells, reduced yield and cell contamination by other cell types) but are often less representative of the *in vivo* characteristics due to loss of significant barrier function during passage (55, 56). A comprehensive list of endothelial cell lines frequently employed in BBB model systems has been reviewed (57, 58). Importantly, data obtained from *in vitro* models must take into account any differences in species of the cell types used and consider that correlations between *in vitro* and *in vivo* results may diverge. Despite these caveats *in vitro* systems still represent an important resource to improve our understanding of barrier function.

Current *in vitro* model systems can be broadly placed into two categories namely 2 and 3 dimensional (2D and 3D respectively) models. Both have advantages and disadvantages that should of course be taken into consideration before beginning relevant studies. The strength of the 2D model lies in the ability to directly assess barrier function within an endothelial monolayer. In contrast 3D models foster cellular movement and organization of dynamic cell-cell interactions that are more analogous to those that occur *in vivo*.

**2D models**

Two-dimensional cell culture models form the majority of BBB *in vitro* models currently in use and are based on systems developed in the 80's and 90's (24, 56, 59). These models evaluate various aspects of pharmacology, transport, migration and metabolic activity of the BBB. The experimental set up consists of endothelial cells cultured on one side of a cell-culture insert with porous filter membrane (transwell system), allowing formation of a monolayer and induction of cell polarity (see Fig. 1). The functional barrier phenotype is quantified by means of 1) classic transendothelial electrical resistance (TEER) measurements that assess the resistance of the monolayers exposed to electrical current (60) or 2) permeability assays that assess the diffusion/extravasation of labeled proteins and/or cells through the monolayer (60). However, very early models based on endothelial monocultures quickly revealed a limitation of significantly reduced TEER values (10-100Ω) compared to the *in vivo* situation (>1000Ω) and increased permeability due to loss of barrier phenotype in cultured cells (55, 56). When subsequent evidence suggested that glial cells dynamically interact with and regulate barrier properties the development of various approaches to mimic the glial influence was stimulated. Dehouck and colleagues (59) proposed an enhanced 2D model by co-culturing astrocytes on the opposite face of the porous membrane (contact model) allowing closer recreation of the interactions that occur *in vivo* (see Fig. 1). Others cultured astrocytes on the bottom of the well in which the membrane is inserted (25) the so-called non-contact model, or used astrocyte-conditioned media harvested from growing astrocyte cultures (56, 61). Such practices are now commonplace in the field but despite the fact that co-culturing induces higher TEER values than monocultures the degree of induction remains significantly lower than the *in vivo* situation (62).

More recently it was shown that pericytes also increase the integrity of endothelial monolayers (32, 63, 64) and the presence of both astrocytes and pericytes produces optimal induction of the barrier phenotype (50, 65). Indeed such triple cultures are a step closer to the interactions that occur *in vivo* and are currently gaining popularity in the field. Further enhancement of barrier function can be achieved by applying flow-induced shear stress across the monolayer (23, 66, 67). To achieve this a parallel plate flow chamber is frequently used such as employed in studies investigating sequential leukocyte interactions with human microvascular endothelial cells under flow conditions (68, 69). Siddharthan et al also developed a dynamic flow system consisting of a transparent plastic chamber that housed a snapwell insert cultured with human microvascular endothelial cells that could be subjected to peristaltic flow (70).

All of these 2D models have facilitated studies of the contribution of cell-cell interactions and external influences (such as pharmacological compounds and inflammatory cells) to barrier function at a cellular and mechanistic level without interference from other physiological responses. In addition to being able to directly assess barrier function, these models facilitate the investigation of altered signaling pathways and mechanisms within the individual cell compartments. This has been exploited by some researchers to investigate the contribution of astrocytes (50), pericytes (50, 65, 71), neurons (72, 73), neural precursor cells (74, 75) as well immune cells (76) to barrier integrity. Notably these models offer the possibility of reasonably high throughput results and relatively good reproducibility.

However despite providing important information 2D models represent an oversimplified view of the BBB. These models do not take into account the 3-dimensional structure of blood vessels, the complex cellular interactions of cells that comprise the BBB, alteration of cellular properties as a result of lack of one or more cellular constituents or exposure to shear stress. It seems likely that this has only contributed to the discordance between *in vitro* and *in vivo* results. Thus in both the basic research and pharmaceutical worlds the search continues for appropriate 2D models that are more representative of the *in vivo* situation and more reliable for drug screening. Recent reports of mouse, rat and human cell-based BBB models that have high drug permeability correlation coefficients with *in vivo* data, and are possibly suitable for discriminating CNS and non-CNS compounds, may bring that objective one step closer (77-79)

**3D models**

More recently, growing realization that cells behave differently in 2D versus 3D environments have led some researchers to be convinced that more relevant information can be gained from the study of BBB cells in 3D environments. This notion is underlined by the fact that cells of the BBB have a very specific spatial orientation that probably makes a large contribution to the way in which they communicate with each other and maintain barrier cross-talk. This consideration is an important yet regularly overlooked aspect that may contribute to our limited knowledge of barrier properties. Thus new models that offer the advantage of a relatively simplified *in vitro* system but are more representative of (complex) native BBB structure, allowing dynamic movement and reorganization of cells as well as remodeling of the surrounding matrix molecules in response to a changing environment, are sorely needed.

In an effort to better address cellular organization and interactions at the BBB and define the molecular mechanisms underlying these events, a few 3D *in vitro* cell systems have been established.

**3D collagen matrix models**

Endothelial cells undergo morphological changes when grown within a type I collagen matrix that include organization into tube-like structures (80-83) and are accompanied by changes in growth factor receptor profiles and extracellular matrix protein production (84, 85). Some 3D systems involving microvascular endothelial co-culture with astrocytes and neurospheres have also been published (49, 86).

This methodology has been recently adapted to develop and characterize an innovative 3D BBB model (87) that includes all 3 barrier cells and allows them to display their unique morphology as occurs *in vivo* (86, 88). In this model astrocytes, endothelial cells and pericytes are seeded together within the matrix in singe cell suspension. Although endothelial cells cultured alone could form patent vascular structures, improper ABC transporter and junctional protein localization within the tubes highlighted an important limitation of the monocultures (87). It was demonstrated that astrocyte and/or pericyte interactions with the endothelial tubes were required to induce adequate junctional complex localization and endothelial tube polarization in 3D, an observation correlating well with establishment of improved barrier phenotype in 2D co-culture models (50, 65). Astrocyte and pericyte contact with

the vascular structures improved barrier protein localization and effectively induced luminal efflux and accumulation of a P-glycoprotein substrate (87) demonstrating both the presence and activation of an efficient transport mechanism and vascular integrity (89-91). Astrocytes and pericytes also modulated endothelial proliferation and tube morphogenesis. This 3D model is also very dynamic. Hypoxia, a characteristic state of various pathologies that disrupt the BBB (92, 93), modulates both protein localization and cellular interactions within the model system. For example acute hypoxia increased astrocyte coverage of tube structures whereas prolonged hypoxia triggered loss of contacts and vascular swelling (87) as occurs during injury *in vivo* (41). Thus this 3D model displays responses known to occur *in vivo* confirming it is highly representative of the *in vivo* situation. In addition this study supported the concept that co-culturing the 3 cell types is important for cell structure, organization and barrier modulation.

However as with all *in vitro* models there are caveats. Until now, it remains difficult to assess the tightness of the *in vitro* tubes formed. Specific and appropriate cell-cell interaction, TJ expression and transporter activity suggest significant barrier function but it remains very hard to assess directly. There is also the issue of absence of flow that could be an important contributor for the modulation of cell-cell interactions. Nevertheless models such as this offer a new approach to investigate dynamic interactions and the specific contribution of barrier (and non-barrier) cells to complex mechanisms at the BBB during physiological and pathological conditions.

**3D flow systems**

The continuous exposure of endothelial cells to shear stress, generated by blood flow across their apical surfaces, may have important effects on endothelial differentiation and metabolism. Although most *in vitro* BBB models lack the presence of shear stress, some investigators have more fully imitated the physiological environment by exposing microvascular endothelial cells grown in three dimensions to flow (94, 95). Ott et al. developed a hollow fiber cell culture apparatus for this purpose wherein endothelial cells were seeded intraluminally within the capillary-like structure and exposed to continuous peristaltic flow (95). Only a few groups have reported further studies using this model. Janigro and coworkers are continuously developing the system and have gone one step further by seeding the endothelial cells intraluminally while culturing glia on the extraluminal surface of the hollow fiber tube (96, 97). This induced a BBB-specific phenotype with low permeability to intraluminal potassium, negligible extravasation of proteins, and the expression of a glucose transporter. In addition, co-culturing affected the overall morphology of the cells and induced the expression of BBB-specific ion channels (96). The model also supports continuous real-time monitoring of BBB function by measurement of TEER across the barrier via electrodes inserted in the luminal and abluminal compartments and was reported to generate a restrictive paracellular pathway of more than 700Ω (96). Furthermore exposure to controlled pumping rates makes the model suited for the study of endothelial cell responses to a wide range of shear stress. Neuhaus et al. (98) also established a flow based hollow-fiber *in vitro* model of immortalized porcine brain microvascular endothelial cells co-cultured with glia cells. Again monolayer tightness of the model was significantly enhanced by co-culture and surprisingly the system could be maintained for up to 4 months. Cell

attachment and morphology was monitored by environment scanning electron microscopy and showed that the endothelial cells formed a monolayer intraluminally with an elongated morphology presumably due to the influence of pulsatile flow whereas the glial cells grew as a multilayer with their end feet contacting the monolayer. An exciting step for development of these models will be the addition of pericytes to the system.

Dynamic flow systems foster morphological development that better resemble the endothelial phenotype *in situ*, more differentiated endothelial cells compared to conventional culture, significantly increased TEER and decreased permeability as well as a remarkably longer lifetime (96-100). However, although they represent highly innovative approaches, the high cost and technical demands of these models means they have not been adopted in many research labs or used as possible BBB permeability screens. Furthermore, despite high TEER and low permeability values there is still the question of how well the cellular organization within the hollow fibers really correlates to the *in vivo* situation, especially as the cells can be difficult to image. Hopefully in time these systems will become more accessible to the wider community.

### ***In vitro* versus *in vivo* models.**

While it is clear that *in vitro* models represent highly simplified systems of the BBB, their usefulness and power should not be under estimated. In a climate where the use of animals and translation of data from such experiments is highly scrutinized *in vitro* models frequently offer an easy, cost-effective alternative. *In vitro* models also represent a way to study cell-specific contributions, mechanisms and signaling processes in a way that is not possible, or at least very challenging, *in vivo*. However, it is becoming ever more evident that the complexity of currently utilized *in vitro* models needs to be addressed to bring them closer to *in vivo* relevance - single and 2 cell systems seem to no longer fulfill this requirement. Thus there is a great need for development of better, more representative *in vitro* model systems such as those reviewed in this article. Nevertheless as both *in vitro* and *in vivo* models have pros and cons it ultimately seems that the combination of both systems will provide the optimal route to unlock the secrets of induction, maintenance and disruption of BBB function.

## Conclusions

Mechanisms of BBB induction, maintenance and dysfunction are for the large part still a black box. To enable the development of new drugs and therapeutics to combat BBB compromise and brain disease we need to gain more knowledge of how the complex cellular interactions at the BBB modulate different aspects of barrier signaling. It is apparent that *in vitro* models have a significant role to play but new multi-cellular models that are simplified, but still complex, must be developed to bring them closer to *in vivo* relevance. More versatile *in vitro* models, in combination with *in vivo* models, will undoubtedly be the key to understanding the cellular and molecular mechanisms underlying BBB regulation during physiological and pathological situations. Selectively modulating barrier function will remain a significant challenge for all scientists in the BBB field for a while to come.

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